

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
28 August 2003 (28.08.2003)

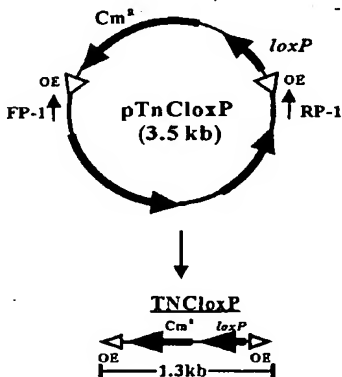
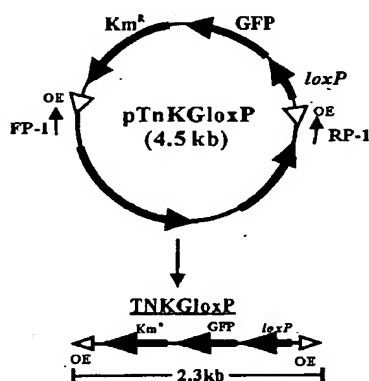
PCT

(10) International Publication Number
WO 03/070955 A1

- (51) International Patent Classification⁷: C12N 15/63, 15/03
- (21) International Application Number: PCT/KR02/02033
- (22) International Filing Date: 31 October 2002 (31.10.2002)
- (25) Filing Language: Korean
- (26) Publication Language: English
- (30) Priority Data:
10-2002-0009647
22 February 2002 (22.02.2002) KR
- (71) Applicant (for all designated States except US): KOREA ADVANCED INSTITUTE OF SCIENCE AND TECHNOLOGY [KR/KR]; 373-1, Kusong-Dong, Yuseong-Ku, 305-701 Daejeon (KR).
- (72) Inventors; and
(75) Inventors/Applicants (for US only): KIM, Sun-Chang [KR/KR]; Dasol Apt. 103-702, Kung-Dong, Yuseong-Ku, 305-335 Daejeon (KR). YU, Byung-Jo [KR/KR]; KAIST Apt. 102-308, 392-3 Kung-Dong, Yuseong-Ku, 305-335 Daejeon (KR).
- (74) Agent: PARK, Jang-Won; 200, Nonhyun-Dong, Kangnam-Ku, 135-010 Seoul (KR).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

[Continued on next page]

(54) Title: CONSTRUCTION OF NOVEL STRAINS CONTAINING MINIMIZING GENOME BY Tn5-COUPLED Cre/loxP EXCISION SYSTEM



(57) Abstract: Disclosed is a method for developing novel strains deleted specific chromosome sites, using transposon and Cre/loxP site-specific recombination by Cre expression vector, wherein the transposon comprises a selectable marker and loxP site. The method comprises the steps of: (1) preparing a transposon comprising a selectable marker and loxP site; (2) inserting the transposon into an optional position of microbial chromosome, and determining the inserted site; (3) integrating two transposons comprising a different selectable marker to one chromosome; (4) deleting a chromosomal site between the two loxP sites by introducing a Cre expression vector into the chromosome of step (3); and (5) repeating steps (3 and 4) for the mutant deleted a part of chromosome, to shorten the chromosome of mutant gradually.

WO 03/070955 A1

BEST AVAILABLE COPY



(84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

6/22/05
10/505328

CONSTRUCTION OF NOVEL STRAINS CONTAINING MINIMIZING GENOME BY Tn5-COUPLED Cre/loxP EXCISION SYSTEM

【Technical field】

5

The present invention relates to strains deleted specific chromosome sites, using transposon with loxP site and Cre/loxP site-specific recombination, and the construction method thereof. More particularly, the present invention relates to a method for constructing novel strains deleted specific chromosome sites, by Cre/loxP site-specific recombination using a transposon comprising a selectable marker and loxP site, and a Cre expression vector.

【Background Art】

15. Even though the technology to delete specific chromosome sites of E. coli by using Cre/loxP site specific recombination is generally known, it has been required to prepare targeting vector and perform PCR (polymerase chain reaction) in every experiment to delete specific chromosome sites in the conventional methods. The random transposition of transposon into the chromosome is also well known. However, it has not been reported that chromosome sites of microorganism can be deleted using transposon together with Cre/loxP site-specific recombination

25

【Detailed Description of the Invention】

The present invention relates to a method of deleting specific chromosome sites, using transposon and Cre/loxP site-specific recombination, in order to improve the conventional method requiring to prepare targeting vector and to perform PCR (polymerase chain
5 reaction) in every experiment.

The present invention relates to a method of preparing strains deleted specific chromosome sites, using transposon and Cre/loxP site-specific recombination. More particularly the present invention relates to a method for developing novel strains deleted specific chromosome
10 sites, by Cre/loxP site-specific recombination using transposon comprising a selectable marker and loxP site and Cre expression vector.

The method for developing novel strains deleted specific chromosome sites comprises the steps of:

- (1) preparing two transposons comprising loxP site and
15 different selectable markers;
- (2) inserting the above two transposons into optional positions of microbial chromosome, respectively, and determining the inserted sites;
- (3) integrating two transposons comprising different
20 selectable markers into one chromosome by P1 phage transduction; and
- (4) transforming a plasmid containing Cre gene into the host strain with the above chromosome and expressing it, to delete a chromosomal site between
25 the two loxP sites of the transposons.

The preparation method can be explained in detail, as follows:

In the above step (1), the above two transposons have different selectable markers. In the Examples of the present invention, TnKGloxP and TnClox were prepared and used as the above two different transposons. TnKGloxP comprises loxP site (SEQ ID NO:4) and Km^R (kanamycin resistant gene, SEQ ID NO:5) as a selectable marker and GFP (Green Fluorescent Protein, SEQ ID NO:6) gene. TnCloxP comprises loxP site (SEQ ID NO:4) and Cm^R (chloramphenicol resistant gene, SEQ ID NO:7) as a selectable marker. The above two transposons have outer end transposase recognition sequences (OE sequence) comprising 19 base pairs at each terminus, which have SEQ ID NO:3 (5'-ctgtctcttatacacatct-3') and its reverse-complementary sequence (5'-agatgtgtataagagacag-3'), respectively.

In other words, transposon TnKGloxP has outer end transposase recognition sequences (OE sequence) comprising 19 base pairs at each terminus, loxP site, Km^R as a selectable marker and GFP gene. Transposon TnCloxP has outer end transposase recognition sequences (OE sequence) comprising 19 base pairs at each terminus, loxP site and Cm^R as a selectable marker. In the above transposons, the length and the sequence of the transposon can vary depending on the vector used for the preparation of transposon except the OE sequence at each terminus and loxP site, which are essential for recombination.

The above transposons, TnKGloxP and TnCloxP can also be obtained by PCR from the pTnKGloxP and pTnCloxP vectors,

respectively (see Figure 1).

According to one of the preferable examples, the above transposons TnKGloxP and TnCloxP may be prepared, as follow:

First, the preparation procedures of the above transposon

5 TnKGloxP include the steps of:

- preparing a new vector pKGloxP by inserting GFP gene into pKKloxP vector comprising the linear Km^R and loxP by using ligase;
- separating a DNA fragment comprising Km^R, GFP and loxP
10 sites by treating pKGloxP vector with restriction enzyme;
- preparing pTnKGloxP vector by inserting the above separated DNA fragment into the linear pMODTM<MCS> vector by using ligase; and
- performing PCR of the above pTnKGloxP vector.

15 Also, the preparation procedures of the above transposon, TnCloxP include the steps of:

- separating a DNA fragment comprising Cm^R and loxP sites by treating pKGloxP vector comprising Cm^R and loxP sites with restriction enzyme;
- 20 - preparing pTnCloxP vector by inserting the above separated DNA fragment into the linear pMODTM<MCS> vector by using ligase; and
- performing PCR of the above pTnCloxP vector.

The following base sequences of the prepared Transposons
25 TnKGloxP and TnCloxP according to the above methods, are shown as

SEQ ID NO:1 and SEQ ID NO:2, respectively.

TnKGloxP base sequence

1 attcaggctg cgcaactgtt gggaagggcg atcgggtcgg gcctcttcgc tattacgcca
 5 61 ***gctgtctctt atacacatct*** caaccatcat cgatgaattc gagctcggtg cccgggttga
 || ← OE sequence → ||
 121 actcgggatc ttgcggccgc aaaaattaaa aatgaagttt ***tgacggtatc gaaccccaga***
 || ← Km^R → ||
 181 gtcccgtca gaagaactcg tcaagaaggc gatagaaggc gatgcgctgc gaatcgggag
 10 241 cggcgatacc gtaaagcacg aggaagcggg cagcccattc gccgccaagc tcttcagcaa
 301 tatcacgggt agccaacgct atgtcctgat agcgggtccg cacaccacgc cggccacagt
 361 cgatgaatcc agaaaagcgg ccattttcca ccatgatatt cggcaagcag gcacgcgcat
 421 gggtcacgac gagatcctcg ccgtcgggca tccgcgcctt gagcctggcg aacagttcgg
 481 ctggcgcgag cccctgatgc tcttcgtcca gatcatcctg atcgacaaga ccgggttcca
 15 541 tccgagtacg tgctcgctcg atgcgatgtt tcgcttggtg gtcgaatggg caggtagccg
 601 gatcaagcgt atgcagccgc cgcattgcat cagccatgat ggatacttc tcggcaggag
 661 caagtgaga tgacaggaga tctgccccg gcacttcgcc caatagcagc cagtcccttc
 721 ccgcttcagt gacaacgctg agcacagctg cgcaaggaac gcccgctgtg gccagccacg
 781 atagccgcgc tgctcgtct tggagttcat tcagggcacc ggacaggtcg gtcttgacaa
 20 841 aaagaaccgg gcgccccgctg gctgacagcc ggaacacggc ggcacagag cagccgattg
 901 tctgttgtg ccagtcatag ccgaatagcc tctccacca agcggccgga gaacctgcgt
 961 gcaatccatc ttgttcaatc atgcgaaacg atcctcatcc tgtctcttga tccactagat
 1021 tattgaagca ttatcagggt ttattgtctc atgagcggat acatatttga atgtatttag
 1081 aaaaataaac aaataggggt tccgcgcaca ttccccgaa aagtgccacc tgcacgatg
 25 || ← Km^R → ||
 1141 aattgatccg aagttcctat tctctagaaa gtaggaac ttogaattgt cgacaagctt
 1201 gatctggctt atcgaaatta atacgactca ctataggag accggaattc ***attatttga***
 || ← GFP → ||
 1261 gagctcatcc atgccatgtg taatcccagc agcagttaca aactcaagaa ggaccatgtg

1321 gtcacgcttt tcgttgggat ctttcgaaag ggcagattgt gtcgacaggt aatgggtgtc
 1381 tggtaaaagg acagggccat cgccaattgg agtattttgt tgataatggt ctgctagtgtg
 1441 aacggatcca tcttcaatgt tgtggcgaat ttgaagta gctttgattc cattcttttg
 1501 ttgtctgcc gtgatgtata cattgtgtga gttatagtgt tactcgagtt tgtgtccgag
 5 1561 aatggttcca tcttctttaa aatcaatacc tttaactcg atacgattaa caagggtatc
 1621 accttcaaac ttgacttcag cagcgcgtct gtagttcccg tcacttttga aagatatagt
 1681 gcgttctgt acataacctt cgggcatggc actcttgaaa aagtcatgcc gtttcatatg
 1741 atccggataa cgggaaaagc attgaacacc ataagagaaa gtagtgacaa gtgttgacca
 1801 tggaacaggt agttttccag tagtgcaaat aaatttaagg gtaagtttc cgtatgtgc
 10 1861 atcaccttca ccctctccac tgacagaaaa ttgtgcca ttaacatcac catctaattc
 1921 aacaagaatt gggacaactc cagtgaagaa ttcttctctt ttactcattt ttctaccgg
 1981 taccgggga tctctagag tcgacctgca ggcattgcaag ctggcgtaa tcattggtcat
 2041 agctgtttcc tgtgtgaaat tgttatccgc tcaaatcc acacaacata cgagccggaa
 2101 gcataaagt taaagcctgg ggtgcctaag gagtgagcta actcacatta attgcgttgc
 15 2161 gctcactgcc cgctttccag tcgggaaatc caagggcgaa ttcgagctcg gtaccgggcc

 GFP → ||
 2221 cccctcgag ggaccttaata *acttcgtata gcatacatta tacgaagta* tattaagggt

 || ← loxP site → ||
 2281 tccggtacct ctgagtaga cctctagagt cgacctgcag gcatgcaagc ttcagggttg
 20 2341 *agatgtgtat aagagacagc* tgcattaatg aatcgccaa cgcgcgggga gaggcggtt

 || ← OE sequence → ||
 2401 gcgtattggg cgctctccg ctctctgct cactgac

TnCloxP base sequence

25 1 attcaggctg cgcaactgtt gggaagggcg atcgggtcgg gcctcttcgc tattacgcca
 61 *gctgtctctt atacacatct* caaccatcat cgatgaattc gagctcggtta ccgcaaaaat

 || ← OE sequence → || Cm^R
 121 taaaaatgaa gttttaaatc aatctaaagt atatatgagt aaacttggtc tgacagttac
 181 caatgcttaa tcagtggagc accaataact gccttaaaaa aattacgccc cgccctgcca

241 **ctcatgcag tactgttgta atcattaag cattctgccg acatggaagc catcacagac**
 301 **ggcatgatga acctgaatcg ccagcggcat cagcaccttg tcgccttgcg tataatattt**
 361 **gccccatggtg aaaacggggg cgaagaagtt gtccatattg gccacgttta aatcaaaact**
 421 **ggtgaaactc acccagggat tggctgagac gaaaaacata ttctcaataa accctttagg**
 5 481 **gaaataggcc aggttttcac cgtaacacgc cacatcttgc gaatatatgt gtagaaactg**
 541 **ccggaaatcg tcgtggtatt cactccagag cgatgaaaac gttcagttt gtcgatggaa**
 601 **aacggtgtaa caagggtgaa cactatccca taccaccagc tcaccgtctt tcattgccat**
 661 **acggaatttc ggatgagcat tcatacggcg ggcaagaatg tgaataaagg ccggataaaa**
 721 **cttgtgctta ttttcttta cggctcttaa aaaggccgta atatccagct gaacggtctg**
 10 781 **gttataggta cattgagcaa ctgactgaaa tgcctcaaaa tgttctttac gatgccattg**
 841 **ggatatatca acggtggtat atccagtcat tttttctcc atttagctt ccttagctcc**
 901 **tgaaaatctc gataactcaa aaaatacgcc cggtagtcat cttatttcat tatggtgaaa**
 961 **gttggaacct ctacgtgcc gatcaacgtc tcatttcgc caaaagttgg cccagggctt**
 1021 **cccgggatca acagggacac caggatttat ttattctgcg aagtgatctt ccgtcacagg**
 15 1081 **tatttattcg gcgcaaagtg cgtcgggtga tgctgccaac ttactgattt agtgatgat**
 1141 **ggtgttttgg aggtgctcca gtggcttctg ttctatcag catcgatgaa ttgatccgaa**
 Cm^R → ||
 1201 **gttctattc tctagaaagt ataggaactt cgaattgtcg acaagcttga tctggcttat**
 1261 **cgaattaat acgactcact ataggagagc cggaattcga gctcgtacc gggccccccc**
 20 1321 **tcgagggacc taataacttc gtatagcata cattatacga agttatatta agatcctcta**
 || ← loxP site → ||
 1381 **gagtcgacct gcaggcatgc aagcttcagg gttgagatgt gtataagaga cagctgcatt**
 || ← OE sequences → ||
 1441 **aatgaatcgg ccaacgcgcg gggagaggcg gttgcgtat tggcgctct tccgcttct**
 25 1501 **cgctcactga c**

As mentioned above, the base sequence and the length of the remainder parts, other than loxP site, OE sequence, Km^R gene, GFP gene and Cm^R gene sites, may vary depending on the vector used for

the preparation of transposon. The deletion, insertion, and/or substitution of one or more bases thereof does not change the function of transposon, if the base sequences of the loxP site, OE sequence, selectable marker (Km^R /GFP or Cm^R) are all included and preserved.

5 Further, loxP site is located between the outer end transposase recognition sequences on both termini, and not inserted in the middle or the inside of the selectable marker, wherein it does not matter that loxP site is located in 3' side or 5' side of the selectable marker.

In the above step (2), transposases are added to the above two
10 transposons TnKGloxP and TnCloxP, respectively, to form transposome, respectively; the transposomes including different transposons are transferred to different microorganisms using electrophoration; each transposon is inserted into the random site of the chromosomes of the microorganism; mutant microorganism inserted with the above
15 transposon is selected; and the insertion site of the transposon in the selected mutants is identified. Since the random inserting function of the transposase can be activated by Mg^{2+} ion, the formation of transposome is carried out in the absence of Mg^{2+} ion, and the random insertion of transposon in the microorganism is carried out in the
20 presence of Mg^{2+} ion.

Also, since the two transposons include kanamycin resistant gene and chloramphenicol resistant gene, respectively, and thus, they are resistant against the above two antibiotics, the strains with the respective transposon can be selected by cultivating them in the
25 kanamycin- or chloramphenicol-containing media after inserting the

above transposon thereto. The random insertion of transposon can be identified by Southern blot analysis, and the position of the inserted transposon can be confirmed by arbitrary PCR.

In the above step (3), a strain inserted with one transposon at one of the termini of the chromosomal site to be deleted and a strain inserted with other kind of transposon are collected from the strains in which the position of transposon have been identified. Using one of the selected strains as the donor and the other as recipient, the above two transposons are located on both ends of the chromosomal site to be deleted by general P1 phage transduction method, wherein the loxP sites must be located in the same direction.

In the above step (4), Cre gene is transferred into the mutant strain containing transposons on both ends of the chromosomal site to be deleted by transferring the pTSCre expression vector into the above mutant strain. Since the transcription of the Cre gene in the pTSCre expression vector is controlled by tetracycline promoter (Ptet), Cre Recombinase is synthesized by expressing Cre gene by cultivating the mutant strain introduced with the above pTSCre expression vector in the medium containing chlorotetracycline. Cre Recombinase in this synthesis helps to remove the chromosomal site containing two loxP sites in either end by specifically cleaving the above two loxP sites and ligating the cleavage sites.

In addition, the preparation methods according to the present invention can additionally include the step of repeatedly performing steps (3) and (4) on the mutant strains containing deletions of a partial

chromosomal site to reduce the size of the chromosome by degrees. In other words, by randomly selecting two from the mutants containing deletions of the above specific chromosomal sites; fusing the chromosomes of the two selected mutants into one chromosome by P1 phage transduction to obtaining a new strain containing an extended chromosomal deletion site as large as the deleted sites of the above two mutants' chromosomes; and further extending the deleted site of the chromosome by continuously performing P1 phage transduction between the obtained new strain repeatedly and another mutant to reduce the size of the chromosome of mutant by degrees, a mutant with a larger deleted site of chromosome is obtained. In the continuous P1 phage transduction, in order to efficiently select the mutant with the desired deletion site, selectable marker acting as P1 recipient is removed by homologous recombination.

In the present invention, *E. coli* was used as the above microorganism, and Tn5 was used as the above transposon. It has been reported that Tn5 can be inserted into an random site of the chromosome (Berg, D. D., and M. M. Howe. 1989. Mobile DNA. American Society for Microbiology, Washington, D.C.), and Cre DNA recombinase recognizes the two loxP sites to catalyze the DNA recombination reaction between them (Abremski, K., HoessR., and Sternberg, N. 1984. Studies on the properties of P1 site-specific recombination. Cell 32, 1301-1311).

Also, P1 phage is known to have a function transferring a part of the chromosome of the host microorganism to other microorganism

(Watanabe, T., Furuse, C., and Sakaizumi, S. 1968. Transduction of various R factors by phage P1 in *Escherichia coli* and by phage P22 in *Salmonella typhimurium*). Therefore, the present invention develops a method of novel mutant microorganism containing deletion of a partial
5 chromosomal site between two loxP sites, by inserting the transposon with loxP site into an random site of the microorganism chromosome, positioning two loxP sites inside a single chromosome in the same direction by using P1 phage transformation, and introducing Cre expressing vector to express Cre DNA Recombinase.

10 The invention will be further illustrated by the following examples. It will be apparent to those having conventional knowledge in the field that these examples are given only to explain the present invention more clearly, but the invention is not limited to the examples given.

15 **【Brief Description of Drawings】**

Figure 1 shows the preparation processes and the structures of transposons TnKGloxP and TnCloxP.

Figure 2 shows the steps of preparing *E. coli* in which a specific chromosomal site is deleted by using transposons TnKGloxP and
20 TnCloxP.

Figur3 3A and 3B show the sites that can be inserted with TnKGloxP and TnCloxP in the *E. coli* genome.

Figure 4A through 4D show the method of deleting specific sites of *E. coli* chromosome by using transposons TnKGloxP and TnCloxP
25 inserted in various sites and the PCR results of the deleted

chromosome.

EXAMPLE 1

Preparation of transposons TnKGloxP and TnCloxP that 5 can be inserted into random site in the E.coli chromosome

Figure 1 represents a linear transposons TnKGloxP and TnCloxP, wherein TnKGloxP contains Km^R, GFP (pGFPuv, Clontech, Palo Alto, CA) and loxP, and TnCloxP contains Cm^R and loxP sites. The above two transposons have outer end transposase recognition
10 sequences (O Epicentre technologies, Madison, WI) comprising 19 base pairs at each terminus. As can be seen in Figure 1, the above transposons TnKGloxP and TnCloxP can be obtained by PCR from pTnKGloxP and pTnCloxP vectors, respectively.

The above pTnKGloxP vector was prepared by the following
15 method. First, after GFP gene obtained from PCR was cleaved by EcoRI restriction enzyme (New England Biolabs, Beverly, MA), the GFP gene was inserted into vector pKKloxP (Michael D. Koob, et al, 1994, In vivo excision and amplification of large segment of the Escherichia coli genome, Nucleic Acids Research 22(12),2392-2398) which was cleaved
20 by EcoRI restriction enzyme to be a linear form and with Km^R and loxP sites, by using ligase (New England Biolabs, Beverly, MA), to obtain a novel vector. The novel vector was named as pKGloxP.

After separating the DNA fragment of 2.2 kb having Km^R gene, GFP gene and loxP site by reacting the above pKGloxP with NotI/XbaI
25 restriction enzyme (New England Biolabs, Beverly, MA), the separated

DNA fragment was inserted by using ligase into linear pMODTM<MCS> (Epicentre technologies, Madison, WI) treated with BamHI restriction enzyme, to obtain vector pTnKGloxP (see Figure 1).

The above pTnCloxP vector was prepared as follows: After
5 separating 1.2 kb sized DNA fragment having Cm^R and loxP sites by cleaving pKCloxP (Michael D. Koob, et al, 1994, In vivo excision and amplification of large segment of the Escherichia coli genome, Nucleic Acids Research 22(12), 2392-2398) vector containing Cm^R and loxP sites with NotI and BamHI restriction enzymes (New England Biolabs,
10 Beverly, MA), the above DNA fragment was inserted by using ligase into linear pMODTM<MCS> vector treated with BamHI restriction enzyme to prepare pTnCloxP vector (Figure 1).

Transposon TnKGloxP and TnCloxP were prepared from the above pTnKGloxP and pTnCloxP vectors, respectively by PCR. The
15 primers used in the above PCR are pMOD<MCS>FP-1(SEQ ID NO:8) and pMOD<MCS>RP-1(SEQ ID NO:9) with the following base sequence.

pMOD<MCS>FP-1 : 5' ATT CAG GCT GCG CAA CTGT -3'

20 pMOD<MCS>RP-1 : 5' -TCAGTGAGCGAGGAAGCGGAAG-
3'

EXAMPLE 2

Preparation of two kinds of *E. coli* mutant libraries inserted

with transposons TnKGloxP and TnCloxP into random sites of *E. coli* chromosome and identification of the insertion site

After reacting 500 ng of TnKGloxP and 500 ng of TnCloxP with 10 μ l of Tn5 transposase, respectively, and adding DDW (Double Distilled Water) to make the total volume of the solution to 20 μ l, each transposome was formed by reacting at 25 °C for 30 min. To inhibit the random insertion function of the transposase, the above reaction was carried out in the absence of Mg^{2+} ion. One microliter of the transposome was transferred into *E. coli* strain MG1655 (Seoul National University Biological Science Division, Dr. J. H. Noh) by conventional electrophoration method [Bio-RAD, Bacterial electro-transformation and Plus Controller Instruction Manual, Cat.No 165-2098 ; Thompson, JR, et al. An improved protocol for the preparation of yeast cells for transformation by electrophoration. Yeast 14, 565-571 (1998) ; Grant, SG, et al. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA 87, 4645-4649 (1990)], in the presence of Mg^{2+} ion. LB medium (tryptone 1%, yeast extract 0.5%, NaCl 0.5%) was used for the cultivation of the strain, wherein a small amount of Mg^{2+} ion was contained.

Therefore, the random insertion function of transposase can be activated by Mg^{2+} ion inside *E. coli* cells, and transposome can be inserted into the *E. coli* chromosome at random sites. Since the *E. coli* mutant comprising the above two transposons inserted have kanamycin or chloramphenicol resistance due to Km^R or Cm^R gene in the

transposons, they were selected in the kanamycin or chloramphenicol media. Each transposon inserted into the chromosome of E.coli MG1655 was identified by Southern blot analysis.

The conditions for Southern blot analysis were as follows: After
5 separating the chromosomal DNA from the E. coli mutant in which transposon was inserted and cleaving the chromosomal DNA by using ClaI restriction enzyme (New England Biolabs, Beverly, MA), electrophoresis was performed on 1 % agarose gel. DNA in the agarose gel was transferred onto Hybond N+ membrane (Amersham)
10 and the transferred DNA was blotted by using ³²P-labeled Km^R or Cm^R gene as probe. The transposon insertion site was confirmed by arbitrary PCR (Caetano-Annoles, G. 1993. Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl., 3, 85-92.).

DNA around the transposon insertion site was amplified using
15 Tn5 insertion sequence specific primer and the primer that can arbitrarily bind to the chromosomal DNA at the outer side of transposon. The above arbitrary PCR was composed of two steps:

In the first step, single strand DNA containing the end sequence of transposon and the outer sequence thereof by using transposon
20 specific primer Tn5Ext (5' AGCATACATTATACGAAGTTATATTAAG-3', synthesized by Genotech), and subsequently, the primer Arb1 (5' TTGAGCGATAGACGTACGATNNNNNNNNNGATAT-3', synthesized by Genotech) binding to nonspecific site was bound to an nonspecific site of the above synthesized single strand DNA, to

synthesize double strand DNA.

In the second step, the above synthesized double strand DNA was amplified in large scale by using transposon specific primer Tn5Int and primer Arb2 (5' TTGAGCGATAGACGTACGAT-3', synthesized by Genotech) whose base sequence is identical to 25 sequence of 3' end of Arb1. The amplified DNA was separated from the agarose gel by using Qiaquick spin PCR purification kit (Quiagen), the base sequence of the above separated DNA was analyzed using primer Tn5Int (5' TCGACCTGCAGGCATGCAAGCTTCA-3', synthesized by Genotech), and the insertion site was identified by comparing the above analysis result with Gene Bank DNA sequence by using BLAST program. The identified insertion sites of TnKGloxP and TnCloxP by the above methods are shown in Figure 3.

15 EXAMPLE 3

Construction of *E. coli* mutant containing two transposons on the chromosome by P1 phage transduction

Two mutants with the two different transposons at one end and the other end of the specific chromosomal site to be removed, respectively, were selected from the TnKGloxP mutant library and TnCloxP mutant library prepared according to Example 2. Then, the two chromosomes with the different transposons were integrated in a single chromosome by P1 phage transduction so that one transposons should be located on one end of the chromosomal site to be removed

and the other transposon should be located on the other end. The loxP was positioned in the same direction, and P1 phage transduction was carried out by following a well-known protocol (Miller, J., H., editors, 1992, A short Course in Bacterial Genetics; A Laboratory Manual and
5 Handbook for Escherichia coli and Related Bacteria, New York: Cold Spring Harbor).

EXAMPLE 4

**Construction of *E. coli* mutant containing deletion mutation
10 by expressing Cre DNA recombinase by transferring pTSCre expression vector**

pTSCre expression vector (Yoon YG, et al, 1998, Cre/loxP-mediated excision and amplification of the Escherichia coli genome, Gene 14, 89-95) was transferred into the E.coli mutant with TnKGloxP and TnCloxP on either end of the chromosomal site to be deleted.
15 Since the transcription of the Cre gene existing in the pTSCre expression vector is controlled by tetracycline promoter (Ptet), the mutant was cultivated in the medium containing chlorotetracycline at 42 °C, to express Cre recombinase. E.coli mutant with chromosome
20 deletion mutation obtained from the result of expression of Cre DNA recombinase was confirmed by PCR.

In the Examples 3 and 4, a mutant in which TnKGloxP was inserted at b0532 site and a mutant in which TnCloxP was inserted at b0619 site; a mutant in which TnKGloxP was inserted at b2011 site and
25 a mutant in which TnCloxP was inserted at b2073 site; a mutant in

which TnKGloxP was inserted b2829 site and a mutant in which TnCloxP was inserted at b2890 site; and a mutant in which TnKGloxP was inserted at b4271 site and a mutant in which TnCloxP was inserted at b4326 site were used for P1 transduction to express Cre gene. The results are shown in Figures 4A, B, C and D.

EXAMPLE 5

Construction of mutant with extended chromosomal deletion site by repeatedly performing P1 phage transduction method.

The chromosomal deletion site of E. coli mutant containing a specific chromosomal sites were deleted was extended by P1 phage transduction as mentioned in the above Example 3 and 4. First, two mutants were selected from the above specific chromosome site deleted mutants. Using one of them as the donor, and the other as recipient of P1 phage lysate, a new mutant was prepared, in which all of the chromosomal deletion sites of the above two mutants were deleted. Then, this mutant was used again as P1 phage recipient, and the already prepare mutant containing other chromosomal deletion site was used as donor to perform P1 phage transduction continuously and repeatedly. By this method, a chromosomal deletion site of other mutant was repeatedly removed from the chromosome of the above obtained mutant. In the continuous P1 phage transduction, in order to efficiently select the microorganism, selectable marker that acts as P1 recipient was eliminated by homologous recombination.

【Industrial Applicability】

As mentioned above, the present invention related to a method of developing novel *E. coli* strain deleted specific chromosome sites, using transposon and Cre/loxP site-specific recombination. Various sites of *E. coli* chromosome can be selectively and efficiently removed, and library of *E. coli* mutants containing deletions of specific chromosomal sites can be obtained, and a variety of *E. coli* mutants with selectively reduced chromosome can be created by repeatedly performing P1 phage transformation and reducing the chromosome by degrees. By the method of the present invention, nonessential gene for the growth of *E. coli* can be removed, and genetically simplified *E. coli* mutant can be constructed that can be used for the functional research in genomics. Also, fast growing *E. coli* mutant can be selected and used as artificial cell line, since nonessential genes for growth were eliminated. Also, the present invention can be applied to other microorganisms other than *E. coli*, and therefore a variety of mutant microorganisms can be created with selectively reduced chromosomes. Also, in the minimized chromosome prepared according to the method of the present invention, a cassette made by collecting foreign genes related to new metabolism can be constructed and introduced in organisms, to create novel organisms having a variety of useful functions

Claims

1. A transposon TnKGloxP, characterized in comprising outer end transposase recognition sequences having a base sequence of SEQ ID NO: 3 on one end, its reverse-complementary sequence on the other end, loxP site expressed as SEQ ID NO: 4, Km^R gene expressed as SEQ ID NO:5 and GFP gene expressed as SEQ ID NO:6.

2. The transposon TnKGloxP according to Claim 1, chracterized in comprising the base sequence of SEQ ID NO:1.

3. A transposon TnCloxP characterized in comprising outer end transposase recognition sequences having a base sequence of SEQ ID NO: 3 on one end, its reverse-complementary sequence on the other end, loxP site expressed as SEQ ID NO:4 and Cm^R gene expressed as SEQ ID NO:7.

4. The transposon TnCloxP according to Claim 3, characterized in comprising the base sequence SEQ ID NO:2.

5. A method for constructing novel strains containing deletion of a specific chromosomal site, characterized in comprising the steps of:

(1) preparing two transposons comprising outer end transposase recognition sequences, loxP site and different selectable markers;

- (2) inserting the above two transposons, respectively, into random positions of different microbial chromosomes and determining the each inserted sites;
- (3) integrating the two microbial chromosomes by P1 phage transduction to position the two transposons comprising different selectable markers on one chromosome; and
- (4) deleting a chromosomal site between the two loxP sites by expressing Cre gene through Cre expression vector introduced.

10

6. The method for constructing novel strains according to Claim 5, wherein the above two transposons are transposon TnKGloxP according to Claim 1 or 2, or transposon TnCloxP according to Claim 3 or 4.

15

7. The method for constructing novel strains according to Claim 5, wherein,

- transponon TnKGloxP comprising a base sequence of SEQ ID NO:1 is prepared by preparing a vector pKGloxP by inserting GFP gene into a linear pKKloxP vector having Km^R and loxP using ligase; separating a DNA fragment comprising Km^R, GFP and loxP sites by treating the above pKGloxP vector with restriction enzyme; preparing pTnKGloxP vector by inserting the above separated DNA fragment into the linear pMODTM<MCS> vector using ligase; performing PCR of the above pTnKGloxp vector, and

25

- transposon TnCloxP comprising base a sequence of SEQ ID NO: 2 is prepared by separating a DNA fragment comprising Cm^R and loxP sites by treating the above pKGloxP vector comprising Cm^R and loxP sites with restriction enzyme; preparing pTnCloxP vector by
5 inserting the above separated DNA fragment into the linear pMODTM<MCS> vector using ligase; and performing PCR of the above pTnCloxP vector.

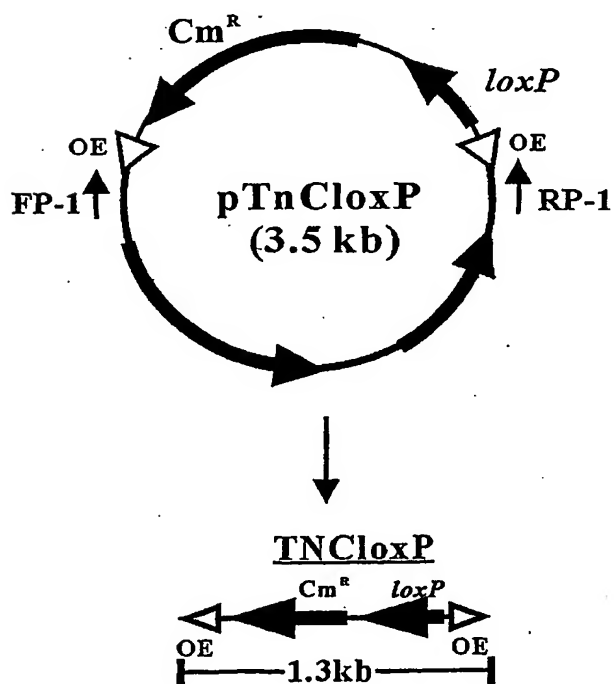
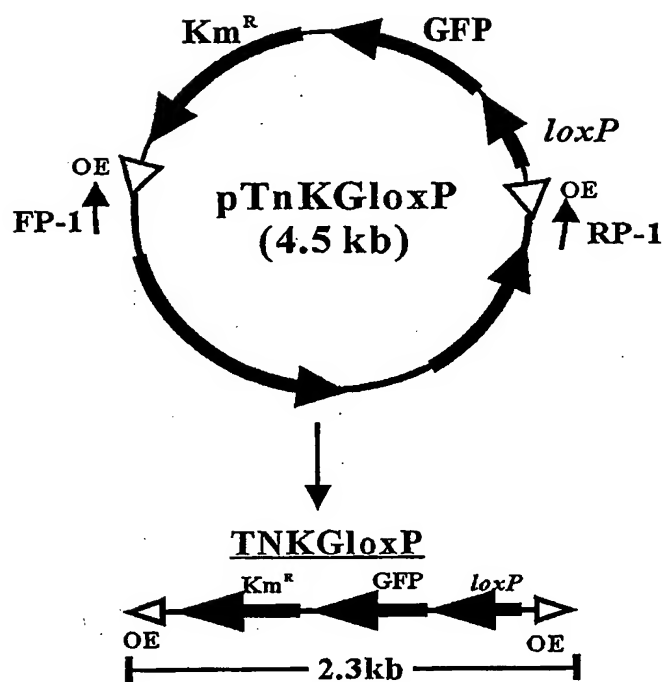
8. The method for constructing novel strains according to Claim
10 5 or 7, characterized in additionally comprising the steps of:

- selecting two mutants from the mutants containing deletion of a specific chromosomal site, and performing P1 phage transduction using one of the selected mutants as the donor and the other as recipient, to constructing a new mutant containing
15 all chromosomal deletion sites of the above two mutant;
- using the above obtained mutant again as P1 phage recipient, and the already prepare mutant containing deletion of a specific chromosomal site as donor to perform P1 phage transduction continuously and repeatedly; and
- 20 - removing the chromosomal deletion site of other donor mutant from the chromosome of the obtained mutant continuously to reduce the chromosome of the obtained mutant by degrees.

10/505328

1/6

FIG. 1



10/505328

2/6

FIG. 2

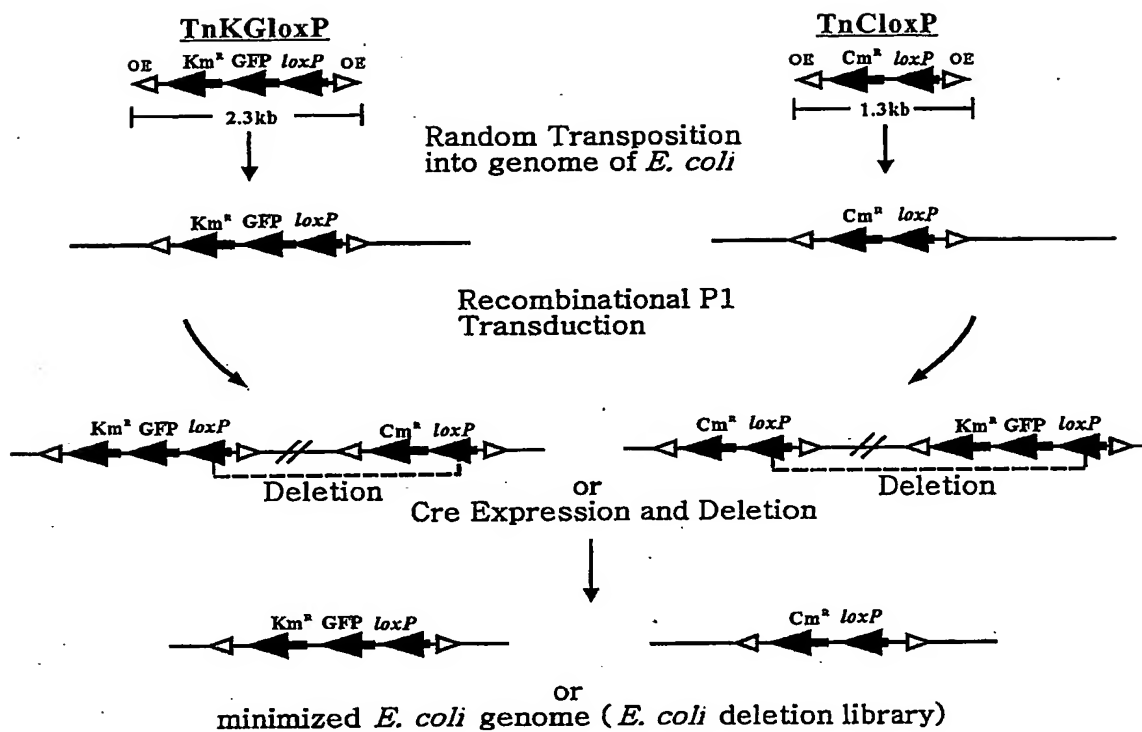


FIG. 3A

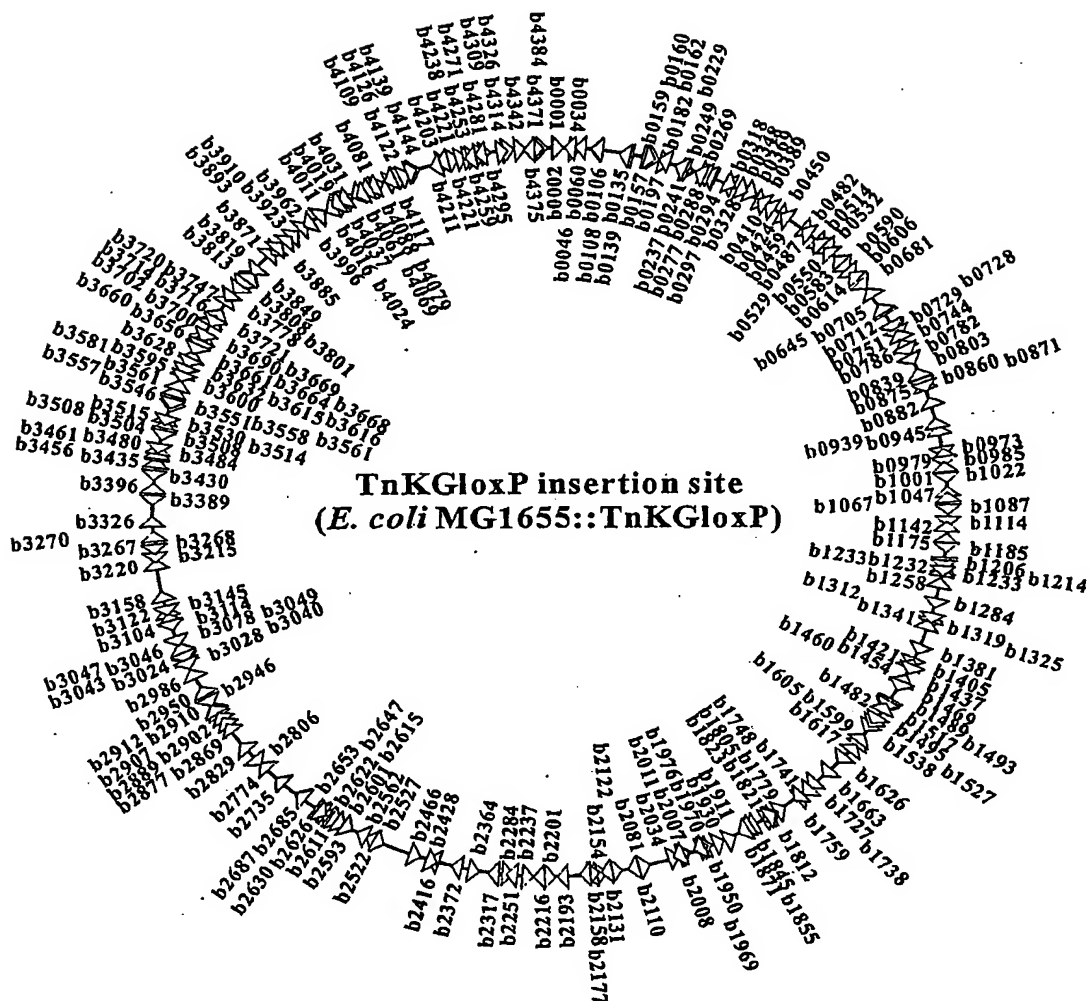
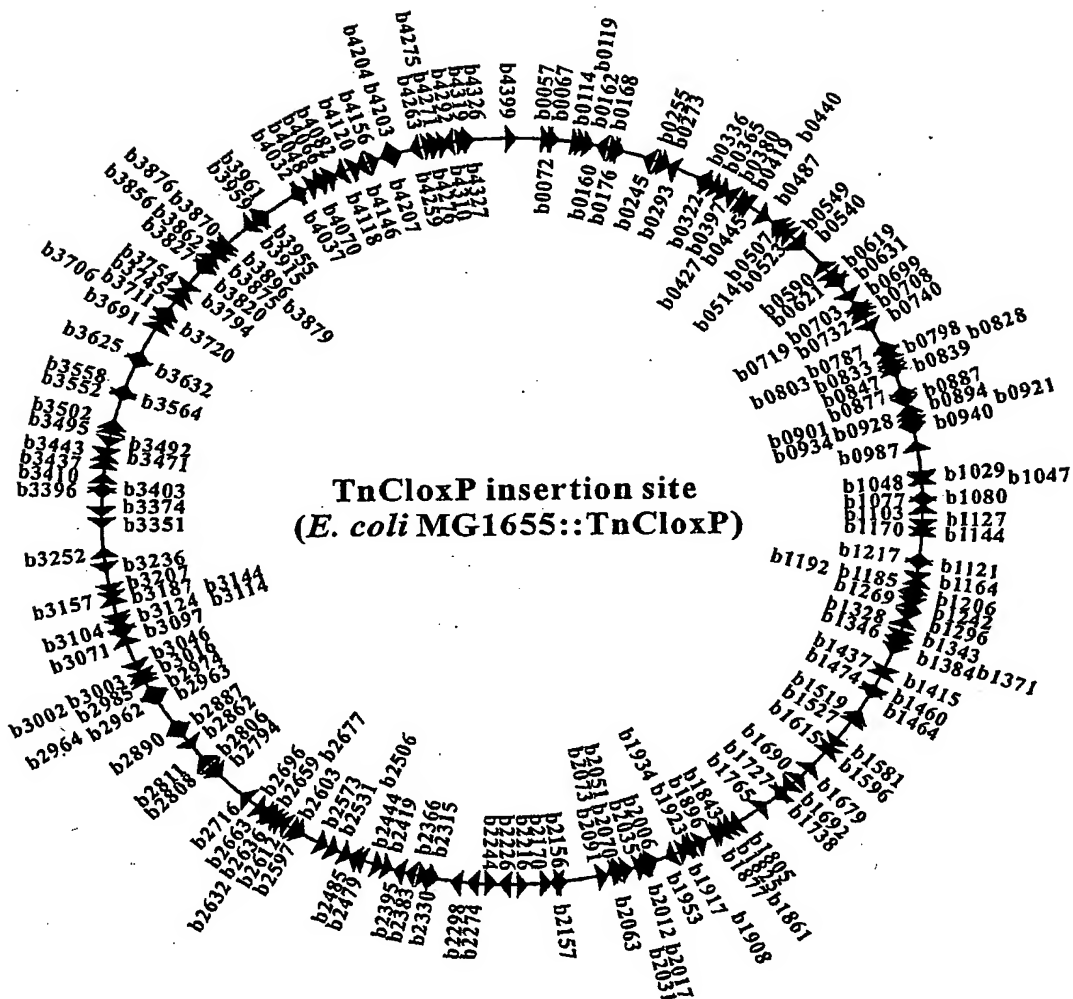


FIG. 3B



5/6

FIG. 4A

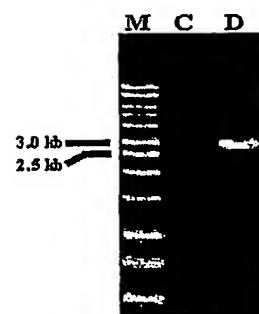
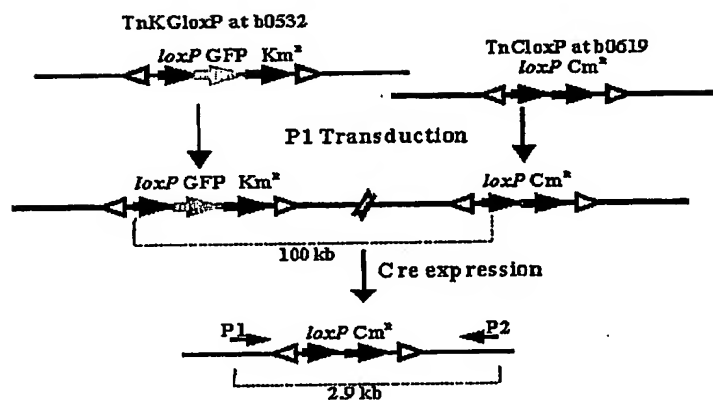
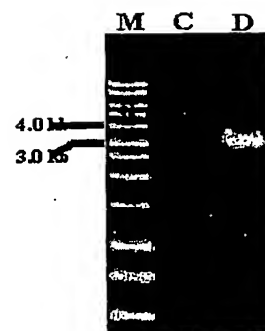
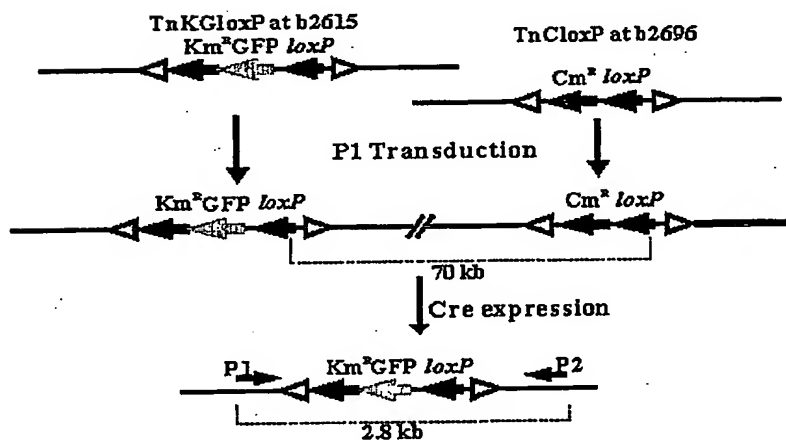


FIG. 4B



10/505328

6/6

FIG. 4C

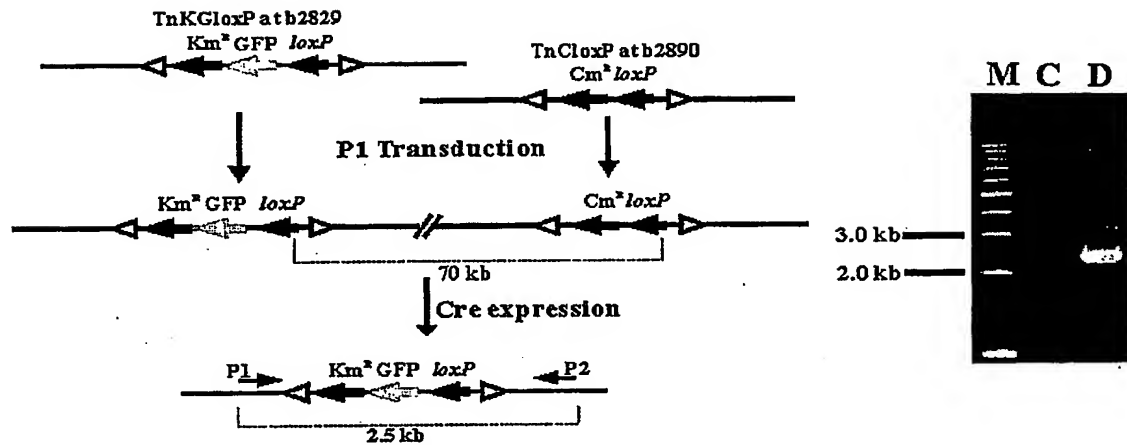
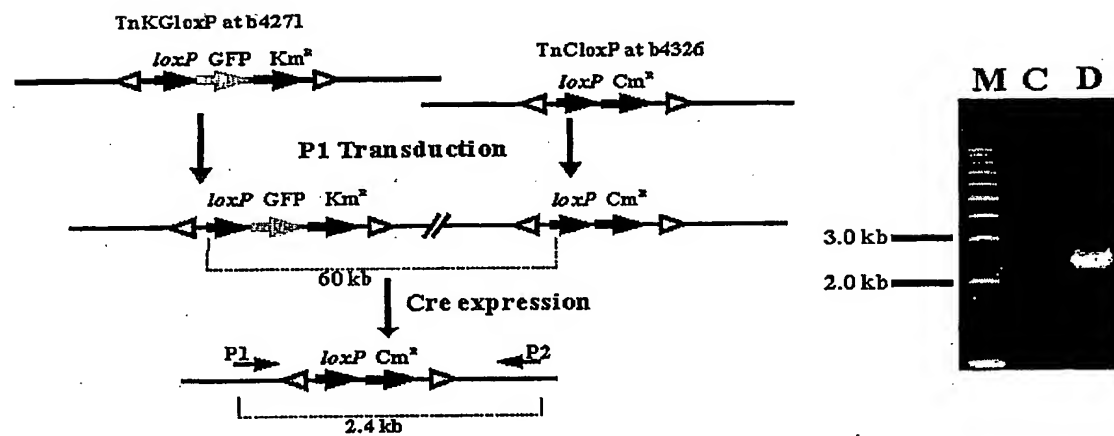


FIG. 4D



DT09 Rec'd PCT/PTO 23 AUG 2004

[Sequence listing]

<110> Korea Advanced institute of Science and Technology
 <120> CONSTRUCTION OF NOVEL STRAINS CONTAINING MINIMIZING
 GENOME BY Tn5-COUPLED Cre/loxP EXCISION SYSTEM
 <160> 13
 <170> KopatentIn 1.71
 <210> 1
 <211> 2437
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> TnKGloxP

<400> 1
 attcaggctg cgcaactgtt gggaagggcg atcgggtcgg gcctcttcgc tattacgcca 60
 gctgtctctt atacacatct caaccatcat cgatgaattc gagctcggta cccgggtga 120
 actgcggatc ttgcggccgc aaaaattaaa aatgaagttt tgacggtatc gaaccccaga 180
 gtcccgtca gaagaactcg tcaagaaggc gatagaaggc gatgcgctgc gaatcgggag 240
 cggcgatacc gtaaagcacg aggaagcggc cagcccatc gccgccaagc tcttcagcaa 300
 tatcacgggt agccaacgct atgtcctgat agcggtcgc cacaccagc cggccacagt 360
 cgatgaatcc agaaaagcgg ccattttcca ccatgatatt cggcaagcag gcatcgccat 420
 gggtcacgac gagatcctcg ccgtcgggca tccgcgcctt gagcctggcg aacagttcgg 480
 ctggcgcgag cccctgatgc tcttcgtcca gatcatcctg atcgacaaga ccggcttcca 540
 tccgagtacg tgctcgctcg atcgatgtt tcgcttggtg gtcgaatggg caggtagccg 600
 gatcaagcgt atgcagccgc cgcattgcat cagccatgat ggatacttgc tcggcaggag 660
 caaggtgaga tgacaggaga tctgccccg gcacttcgcc caatagcagc cagtcccttc 720
 ccgcttcagt gacaacgtcg agcacagctg cgcaaggaaac gcccgctcgt gccagccagc 780
 atagccgcgc tgctcgtct tggagttcat tcagggcacc ggacaggtcg gtcttgacaa 840
 aaagaaccgg gcgcccctgc gctgacagcc ggaacacggc ggcatcagag cagccgattg 900
 tctgtgtgc ccagtcatac ccgaatagcc tctccacca agcggccgga gaacctgcgt 960
 gcaatccatc ttgttaatc atgcgaaacg atcctcatcc tgtctcttga tccactagat 1020
 tattgaagca ttatcaggg ttattgtctc atgagcggat acatatttga atgtatttag 1080

aaaaataaac aaataggggt tccgcgcaca ttccccgaa aagtccacc tgcacgatg	1140
aattgatccg aagttcctat tctctagaaa gtataggaac ttcgaattgt cgacaagctt	1200
gatctggcct atcgaaatta atacgactca ctataggag accggaattc attatttga	1260
gagctcatcc atgcatgtg taatcccagc agcagttaca aactcaagaa ggaccatgtg	1320
gtcacgcttt tcgttgggat ctttcgaaag ggcagattgt gtcgacaggt aatggtgtc	1380
tggtaaaagg acagggccat cgccaattgg agtattttgt tgataatgt ctgctagtgtg	1440
aacggatcca tctcaatgt tgtggcgaat ttgaagtta gctttgattc cattctttg	1500
ttgtctgcc gtgatgtata cattgtgtga gttatagttg tactcgagtt tgtgtccgag	1560
aatgtttcca tcttcttaa aatcaatacc ttttaactc atacgattaa caagggtatc	1620
accttcaaac ttgacttcag cagcgcgtct gtagttccc tcattcttga aagatatagt	1680
gcgttcctgt acataacctt cgggcatggc actcttgaaa aagtcatgcc gttcatatg	1740
atccggataa cgggaaaagc attgaacacc ataagagaaa gtagtgacaa gtgttgcca	1800
tggaaacaggt agttttccag tagtgcaaat aaattaaagg gtaagtttc cgtatgtgc	1860
atcaccttca cctctccac tgacagaaaa ttgtgcccc ttaacatcac catctaattc	1920
aacaagaatt gggacaatic cagtgaagaa ttcttctct ttactcattt ttctaccgg	1980
taccggggga tctctagag tcgacctgca ggcattgcaag cttggcgtaa tcatgtcat	2040
agctgtttcc tgttgaaat tgttatccgc tcacaattcc acacaacata cgagccggaa	2100
gcataaagt taaagcctgg ggtgcctaag gagtgagcta actcacatta attgcgttc	2160
gctcactgcc cgctttccag tcgggaaatc caaggcgaa ttcgagctcg gtaccgggcc	2220
ccccctcag ggaccttaata acttcgtata gcatacatta tacgaagtta tattaagggt	2280
tccggatcct cttagagtaga cctctagagt cgacctgcag gcatgcaagc ttcagggtg	2340
agatgtgtat aagagacagc tgcattaatg aatcggccaa cgcgcgggga gaggcggtt	2400
gcgtattggg cgctcttccg cttcctcgt cactgac	2437

<210> 2
 <211> 1511
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> TnCloxP

<400> 2

attcaggctg cgcaactgtt ggaaggcg atcggcg gcctcttcgc tattacgcca	60
gctgtctctt atacacatct caaccatcat cgatgaattc gagtcggta ccgcaaaaat	120
taaaaatgaa gttttaaatc aatctaaagt atatatgagt aaacttgctc tgacagttac	180
caatgcttaa tcagtgggc accaataact gccttaaaaa aattacgcc cgccctgcca	240
ctcatcgag tactgttgta atcattaag cattctgccg acatggaagc catcacagac	300
ggcatgatga acctgaatcg ccagcggcat cagcaccttg tcgccttcgc tataatattt	360
gccccatgtg aaaacggggg cgaagaagt gtccatattg gccacgtta aatcaaaact	420
ggtgaaactc acccagggat tggctgagac gaaaaacata ttctcaataa acccttagg	480
gaaataggcc aggtttcac cgtaacacgc cacatcttcg gaatatatgt gtagaaactg	540
ccggaaatcg tcgtggtatt cactccagag cgatgaaaac gtttcagttt gctcatgaa	600
aacggtgtaa caagggtgaa cactatccca taccaccgc tcaccgtctt tcattgccat	660
acggaatttc ggatgagcat tcatcaggcg ggcaagaatg tgaataaagg ccggataaaa	720
ctgtgctta ttttctta cggcttta aaaggccgta atatccagct gaacggtctg	780
gttataggta cattagcaa ctgactgaaa tgcctcaaaa tgttcttac gatgccattg	840
ggatatatca acggtggtat atccagtcat ttttctcc atttagctt ccttagctcc	900
tgaaaatctc gataactcaa aaaatacgc cggtagtcat ctatttcatt tatggtgaaa	960
gttggaacct ctacgtgcc gatcaacgtc tcatttcgc caaaagtgg ccaggggctt	1020
cccggatca acagggacac caggatttat ttattctgcg aagtatctt ccgtcacagg	1080
tatttattcg gcgcaaagtg cgtcgggtga tctgccaac ttactgattt agtgatgat	1140
ggtgttttg aggtgctcca gtggtcttg ttctatcag catcgatgaa ttgatccgaa	1200
gttcctattc tctagaaagt ataggaactt cgaattgtc acaagctga tctggcttat	1260
cgaataaat acgactcact ataggagac cggaattcga gtcgggtacc gggccccccc	1320
tcgagggacc taataacttc gtatagcata cattatacga agttatatta agatcctcta	1380
gagtcgacct gcaggcatgc aagcttcagg gttgagatgt gtataagaga cagctgcatt	1440
aatgaatcgg ccaacgcgcg gggagaggcg gtttcgtat tgggcgctct tccgcttct	1500
cgctcactga c	1511

<210> 3
 <211> 19
 <212> DNA
 <213> Artificial Sequence

<220>

<223> OE sequence

<400> 3

ctgtctctta tacacatct

19

<210> 4

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> loxP site

<400> 4

ataacttcgt atagcataca ttatacgaag ttat

34

<210> 5

<211> 996

<212> DNA

<213> Artificial Sequence

<220>

<223> KmR gene

<400> 5

gcaaaaatta aaaatgaagt ttgacggta tcgaaccca gagtcccgct cagaagaact 60

cgtaagaag gcgatagaag gcgatgcgct gcgaatcggg agcggcgata ccgtaaagca 120

cgaggaagcg gtcagcccat tcgccgcaa gctcttcagc aatatcacgg gtagccaacg 180

ctatgtcctg atagcggtc gccacacca gccggccaca gtcgatgaat ccagaaaagc 240

ggccatttc caccatgata ttcggcaagc aggcacgcc atgggtcacg acgagatcct 300

cgccgtcggg catccggcc ttgagcctgg cgaacagttc ggctggcgcg agcccctgat 360

gctctcgtc cagatcatcc tgatcgaca gaccggcttc catccgagta cgtgctcgt 420

cgatgcgatg ttgccttgg tggcgaatg ggcaggtagc cggatcaagc gtatgcagcc 480

gccgcattgc atcagccatg atggatactt tctcggcagg agcaagggtga gatgacagga 540

gatcctgccc cggcacttcg cccaatagca gccagtcct tcccgttca gtgacaacgt 600

cgagcacagc tgcgcaagga acgcccgtcg tggccagcca cgatagccgc gctgcctcgt 660

cttgaggtc attcaggga ccggacaggt cggcttgac aaaaagaacc gggcgcccct 720

gcgctgacag ccggaacagc gcggcatcag agcagccgat tgtctgtgt gccagtcac 780

agccgaatag cctctccacc caagcgccg gagaacctgc gtgcaatcca tctgttcaa 840

tcatgcgaaa cgatcctcat cctgtctctt gatccactag attattgaag catttatcag	900
ggttattgtc tcatgagcgg atacatattt gaatgtattt agaaaaataa acaaataggg	960
gttccgcgca catttccccg aaaagtcca cctgca	996

<210> 6
 <211> 947
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> GFP gene

<400> 6	
attatttga gagctcatcc atgccatgtg taatcccagc agcagttaca aactcaagaa	60
ggaccatgtg gtcacgcctt tcgttgggat ctttcgaaag ggcagattgt gtcgacaggt	120
aatggtgtc tggtaaaagg acagggccat cgccaattgg agtattttgt tgataatgt	180
ctgctagtgt aacggatcca tcticaatgt tgtggcgaat ttgaagta gctttgattc	240
cattctttt ttgtctgcc gtgatgtata cattgtgtga gttatagttg tactcgagtt	300
tgtgtccgag aatgtttcca tcttcttaa aatcaatacc tttaactcg atacgattaa	360
caagggtatc acctcaaac ttgacttcag cacgcgtctt gtagttcccg tcactttga	420
aagatatagt gcgttcctgt acataacctt cgggcatggc actcttgaaa aagtcatgcc	480
gtttcatatg atccggataa cgggaaaagc attgaacacc ataagagaaa gtagtgacaa	540
gtgttgcca tggaacaggt agtttccag tagtgcaaat aaatttaagg gtaagtttc	600
cgtatgttc atcacctca ccctctccac tgacagaaaa ttgtgccca ttaacatcac	660
catctaattc aacaagaatt gggacaactc cagtgaagag ttctctcct ttactcattt	720
tttctaccgg taccgggga tctctagag tcgacctgca ggcattgcaag ctggcgtaa	780
tcatgtcat agctgttcc tgtgtgaaat tttatccgc tcacaattcc acacaacata	840
cgagccggaa gcataaagt taaagcctgg ggtgcctaag gactgagcta actcacatta	900
attgcgttc gctcactgcc cgcttccag tcgggaaatc caagggc	947

<210> 7
 <211> 1069
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> CmR gene

<400> 7
 gcaaaaatta aaaatgaagt tttaaatcaa tctaaagtat atatgagtaa acttggtctg 60
 acagttacca atgcttaatc agtgaggcac caataactgc cttaaaaaaa ttacgccccg 120
 ccctgccact catcgagta ctgttgtaat tcattaagca ttctgccgac atggaagcca 180
 tcacagacgg catgatgaac ctgaatgcc agcggcatca gcacctgtc gccttgcgta 240
 taatatttgc ccatggtgaa aacgggggcg aagaagttgt ccatattggc cacgtttaa 300
 tcaaaactgg tgaaactcac ccagggatig gctgagacga aaaacatatt ctcaataaac 360
 ccttaggga aataggccag gtttcaccg taacacgcca catcttgcga atatattgt 420
 agaaactgcc ggaaatcgtc gtggtattca ctccagagcg atgaaaacgt ttcagttgc 480
 tcatggaaaa cggtgtaaca aggtgaaca ctatccata tcaccagtc accgtcttc 540
 attgccatac ggaatttcgg atgagcattc atcaggcggg caagaatgtg aataaaggcc 600
 ggataaaact tgtcttatt ttctttacg gtcittaaaa aggccgta atccagctga 660
 acggtctggt tataggtaaca ttgagcaact gactgaaatg cctcaaatg ttctttacga 720
 tgccattggg atatatcaac ggtggtatat ccagtattt ttctccat ttagcttcc 780
 ttagctcctg aaaatctga taactcaaaa aatacggccg gtagtgatct tatttcatta 840
 tggtgaaagt tggaacctct tacgtgccga tcaacgtctc atttcgccaa aaagtggcc 900
 cagggtctcc cggtatcaac agggacacca ggattattt attctgcgaa gtgatcttc 960
 gtcacaggtt ttattcggc gcaaagtgcg tcgggtgatg ctgccaactt actgatttag 1020
 tgatgatgg tgttttgag gtgctccagt ggcttctgt tctatcagc 1069

<210> 8
 <211> 19
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> primer-pMOD<MCS>FP-1

<400> 8
 attcaggctg cgcaactgt 19

<210> 9
 <211> 22
 <212> DNA
 <213> Artificial Sequence

<220>
<223> primer-pMOD<MCS>RP-1

<400> 9
tcagtgcgagc aggaagcgga ag 22

<210> 10
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> primer-Tn5Ext

<400> 10
agcatacatt atacgaagtt atattaag 28

<210> 11
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> primer-Arb1

<400> 11
ttgagcgata gacgtacgat nnnnnnnnnn gatat 35

<210> 12
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> primer-Arb2

<400> 12
ttgagcgata gacgtacgat 20

<210> 13
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> primer-Tn5Int

<400> 13
tcgacctgca ggcatgcaag ctcca 25

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.